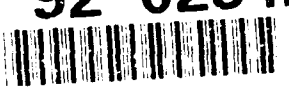
(2)

A TRIDENT SCHOLAR PROJECT REPORT

NO. 176

METALLOPORPHYRIN COMPLEXES AS MODEL COMPOUNDS FOR CYTOCHROME P-450

UNITED STATES NAVAL ACADEMY
ANNAPOLIS, MARYLAND



Metalloporphyrin Complexes as Model Compounds for Cytochrome P-450

A Trident Scholar Project Report

by

Midshipman David L. Blazes, Class of 1991

U.S. Naval Academy

Annapolis, Maryland

Jeffrey Fitzgerald

Assistant Professor Jeffrey P. Fitzgerald

Department of Chemistry

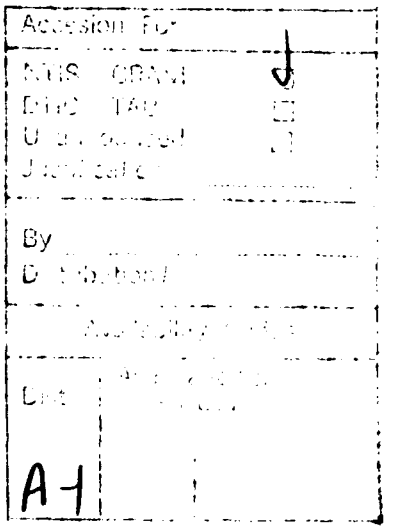
Accepted for Trident Scholar Committee

Francis D Correll

Chair

13 May 1991

Date



REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	13 May 1991	Final 1990/91

4. TITLE AND SUBTITLE

METALLOPORPHYRIN COMPLEXES AS MODEL COMPOUNDS FOR CYTOCHROME P-450

5. FUNDING NUMBERS

6. AUTHOR(S)

David L. Blazes

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Naval Academy, Annapolis, MD

8. PERFORMING ORGANIZATION REPORT NUMBER

U.S.N.A. - TSPR; 176 (1991)

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Accepted by the U.S. Trident Scholar Committee.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

This document has been approved for public release; its distribution is UNLIMITED.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Cytochrome P-450 is the collective name given to a family of heme protein oxygenases found in most eucaryotic organisms. The goals of this project were to explore the role of the thiolate ligand in the catalytic properties of the cytochrome P-450 enzymes and to develop synthetic chemical models of these enzymes. Three possible roles of the thiolate ligand were examined in this study: facilitation of oxygen-oxygen bond cleavage, stabilization of the high valent oxo complex, and moderation of oxygen atom transfer. It was found that the identity of axial ligand did not affect the rate or extent of catalytic oxidation. This provided no accurate comparison for evaluation of the first and third possible roles. It was found that the thiolate ligand actually destabilizes the high valent oxo complex, contrary to the second possible role.

14. SUBJECT TERMS

Cytochrome P-450
Metalloenzymes

15. NUMBER OF PAGES

56

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	

Abstract

Cytochrome P-450 is the collective name given to a family of heme protein oxygenases found in most eucaryotic organisms. In man, these enzymes are found in the liver where they participate in hormone biosynthesis and xenobiotic detoxification. All cytochrome P-450 enzymes contain, at their active site, an iron porphyrin coordinated by a thiolate ligand. The connection between the unusual thiolate ligand and the unique oxidative properties of these enzymes is an unanswered question.

The goals of this project were to explore the role of the thiolate ligand in the catalytic properties of the cytochrome P-450 enzymes and to develop synthetic chemical models of these enzymes. One approach used to study complex molecules like enzymes is to develop "active site" analogues, synthetic molecules that mimic the structural features of the enzyme's active site. If the structure of the model is close to the structure of the enzyme active site, one can expect the model to exhibit catalytic properties similar to those of the enzyme. Once an appropriate model is found, it can be systematically altered and the effects of each alteration observed on the properties of the model. In this way, the function of each portion of a complex molecule can be investigated. This was the approach taken in the following project.

A homologous series of rhodium porphyrin model complexes bearing a variety of axial ligands (thiolate, chloride, imidazole, acetate, triphenylphosphine, etc.) were prepared. Selected physical, chemical, and catalytic properties were examined and compared within the series of rhodium porphyrins in order to understand the role of the thiolate ligand in the oxidative chemistry of cytochrome P-450.

Three possible roles of the thiolate ligand were examined in this study: facilitation of oxygen-oxygen bond cleavage, stabilization of the high valent oxo complex, and moderation of oxygen atom transfer. In relation to these possibilities, several experiments were conducted, and the data compared across the series of rhodium porphyrins differing only in axial ligand. It was found that the identity of axial ligand did not affect the rate or extent of catalytic oxidation. This provided no accurate comparison for evaluation of the first and third possible roles. Interestingly, it was found that the thiolate ligand actually destabilizes the high valent oxo complex, contrary to the second possible role.

The results of this study have raised some interesting questions about cytochrome P-450. Why did nature choose an inherently unstable system for such an important role in the biochemistry of most organisms? How does the unstable iron-sulfur bond contribute to the catalytic oxidation effected by

cytochrome P-450? And finally, how can this powerful reaction be harnessed industrially?

Dedication

This project is dedicated to all those who ascribe to:

"*Anima sano in corpore sano*"

Table of Contents

Abstract....1
Dedication....3
Introduction....6
Enzymes: Biological Catalysts....10
Background on Cytochrome P-450....11
Structure of Cytochrome P-450....13
The Catalytic Cycle of Cytochrome P-450....15
Model Studies....17
Summary of Objectives....20
Statement of Approach....21
Synthesis and Characterization....22
Catalytic Reactions....27
Oxo Complex Formation and Isolation....30
Kinetics of Oxo Formation....33
Electrochemistry....38
Conclusions....40
List of Figures....44
References....45
Appendix 1: Experimental Section....47

Introduction

Chemical reactions are truly involved in every aspect of life. They are as integral to the healing of a wound as they are to the digestion of a roast beef sandwich. Reactions allow plants and animals to grow, to communicate, to reproduce, and to simply exist as entities at all. However, when most people think of chemistry, they picture only limited applications. The chemistry most people think of concerns corrosive acids, poisonous gases, oil spills, and other such "exciting" occurrences. Most people do not wonder how the nylon in their sailing jacket was made, or how that dreaded polyester shirt from the '70's was manufactured. Most people do not wonder about how their bodies digest the fat from McDonald's french fries. It is these everyday aspects of chemistry that the world could not do without.

One chemical process that is applicable to numerous industrial and commercial situations is the selective activation of alkanes. Alkanes are organic (carbon containing) molecules that are relatively inert in a chemical sense; they will react only under extreme conditions and then unselectively.[1] A classic example of this is the simple burning of these molecules as fuel. Because of their low reactivity, alkanes themselves are useless for the production of polymers, commodity chemicals, pharmaceuticals, etc. However, when alkanes become functionalized, that is, when oxygen, nitrogen, or halogen atoms are attached, they become

useful precursors to some of the products mentioned above. If the O, N or X atom could be added selectively, that is to a specific carbon of the alkane, many of these everyday articles could be produced more efficiently. For example, the selective functionalization of hexane could lead to a vastly improved method of nylon production.

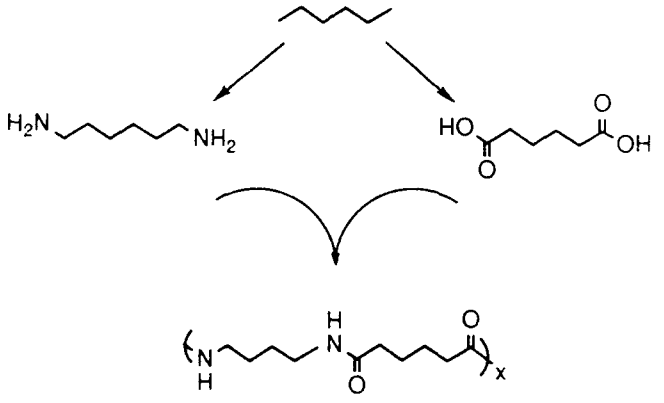


Figure 1: Proposed Synthesis of Nylon Based on Hexane Activation

Currently, the activation of alkanes for the above practical applications cannot be done selectively and can only be achieved through several expensive steps involving time consuming purifications. One method uses halogen gases such as chlorine to oxidize the alkane to a mixture of haloalkanes that must be separated by distillation.[2] The other common method of activating alkanes uses extreme temperatures and pressures to "crack" carbon-carbon bonds and produce alkenes (more reactive relatives of alkanes) that can subsequently be converted into the

desired molecules. Not only are these methods both costly and time consuming, but they are not selective as to the position of functionalization of the alkane. It would be a vast improvement on current chemical technology to devise a method of alkane activation that is inexpensive, easy, and most importantly, selective as to location of functionalization.

Nature provides an excellent model of selective alkane activation under the extremely mild conditions of a biological system (neutral pH and body temperature). More importantly, molecular oxygen (like that in the air) is the oxidizing agent. The biological system that mediates this selective activation is the family of enzymes known collectively as cytochrome P-450.

Cytochrome P-450 catalyzes the oxidation of substrate molecules, including alkanes, with relative ease. The general reaction that cytochrome P-450 catalyzes is:

$$\text{Substrate} + O_2 + 2e^-/2H^+ \rightarrow \text{Substrate(O)} + H_2O$$

Substrate molecules include alkanes, which yield alcohol products, and alkenes, which yield epoxide products. A biochemical example of this oxidation is the synthesis of sex hormones from cholesterol.[3]

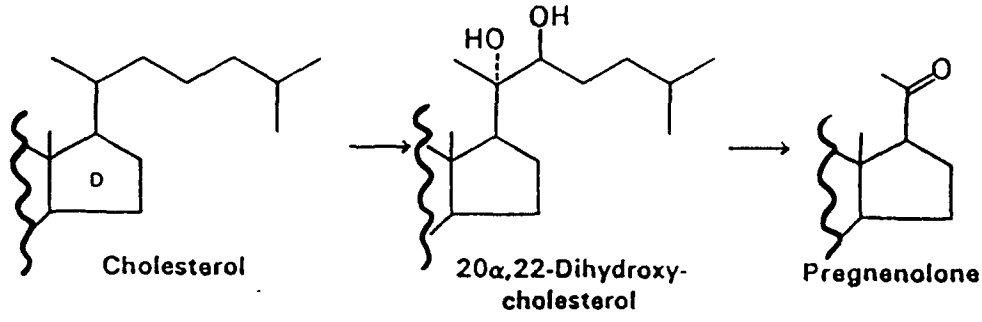


Figure 2: Biosynthesis of Sex Hormones

The benefits of creating a catalyst that would mediate this same type of reaction in a commercial setting would be unbelievable. No longer would cracking towers litter the skylines of industrial cities. A clean and easy method of producing everyday products would revolutionize the current state of the chemical industry.

Enzymes: Biological Catalysts

Enzymes are biological molecules that catalyze, or facilitate, chemical reactions in living systems.[4] They are responsible for the timely response of an organism to stimuli; they make the chemical reactions associated with life practical in the time realm. Different enzymes are involved in muscle contraction and relaxation, in digestion, in DNA replicaton, in oxygen transport, even in thinking. Truly, life could not exist without these important molecules.

Structurally, enzymes are large molecules consisting of a large complicated protein chain and often a much smaller active site prosthetic group. Hemoglobin, for example, consists of a globular protein and an iron porphyrin (heme) prosthetic group.[5] The protein chain, which is composed of amino acid units, coils into a complex arrangement described by the primary, secondary, tertiary, and quaternary structures. For example, the primary structure is simply the exact sequence of amino acid units in the protein chain, whereas the tertiary structure is the three dimensional shape of this protein chain in space. The active site of many enzymes interestingly has the same prosthetic group, a metalloporphyrin, which will be discussed in a following section. The general structure of this metalloporphyrin prosthetic group is constant whether considering chlorophyll, hemoglobin, or cytochrome P-450. The distinguishing factor among these

enzymes is the identity of the coordinated metal in the porphyrin, and the identity of the axial ligands bound to that metal atom. For instance, in hemoglobin, the metal atom is iron and the axial ligand is an imidazole ring from a histidine amino acid, whereas in chlorophyll, the metal atom is magnesium and the axial ligands are two water molecules.[6]

The protein chain, in many enzymes, functions to position substrate molecules in the correct orientation so that a specific chemical reaction might occur between the substrate molecules and the active site of the enzyme. The protein chain thus functions to specify which molecules will be allowed to react with the active site - an ingenious discrimination process. Enzymes can have single active sites or multiple active sites. Hemoglobin has four active sites, while cytochrome P-450 has only one.

Background on Cytochrome P-450

Cytochrome P-450 is a family of powerful oxidative enzymes found in a wide variety of organisms, both very simple and very complex. Contrary to the "cytochrome" portion of the name, these enzymes are not involved in the electron transport mechanism of cellular respiration, as was thought originally. The cytochrome P-450 enzymes, however, do play an important role in the

biochemistry of living organisms, a role that has already been studied and documented.[7]

In living organisms, cytochrome P-450 is involved in both the catabolic and anabolic aspects of metabolism. It is in these roles that cytochrome P-450 demonstrates its unusual oxidative properties. As an anabolic catalyst, or one that helps to build molecules, cytochrome P-450 functions to synthesize sex hormones from cholesterol molecules through a series of difficult chemical reactions. Cytochrome P-450 effects these oxidative reactions with relative ease. As a catabolic catalyst, or one that breaks down molecules, cytochrome P-450 assists in the metabolism of drugs and other xenobiotics to non-toxic, excretable products.[8] Because of its metabolic role, there are large concentrations of this enzyme found in the liver. The powerful oxidative chemistry of cytochrome P-450 is of interest to synthetic industrial chemistry. The reactions it can mediate would allow greater production of chemical goods at lower costs. For example, nylon, produced in the millions of tons a year, would be easier to synthesize if the chemistry of cytochrome P-450 could be harnessed.

Structure of Cytochrome P-450

Cytochrome P-450 is a large globular protein of approximately 50,000 atomic mass units (Daltons). The tertiary structure of the enzyme holds a single active site. The surrounding protein functions to position substrate molecules in the correct three dimensional orientation for the reaction to be possible. The size and complexity of the protein chain is shown in *Figure 3* below.[9] Note the size of the protein in relation to that of the active site, circled in the picture below.

Figure 3: Enzyme Structure

The active site of cytochrome P-450, shown below in *Figure 4*, contains an iron porphyrin.[10] The amino acid cysteine is attached

to the iron atom in cytochrome P-450 in the axial ligand position by an unusual iron to sulfur-anion bond. This thiolate ligand is thought to contribute to the unique oxidative power of cytochrome P-450 and will be the focus of this study. The iron porphyrin, as well as the thiolate ligand, is constant in all cytochrome P-450 enzymes, regardless of where they are found.[11]

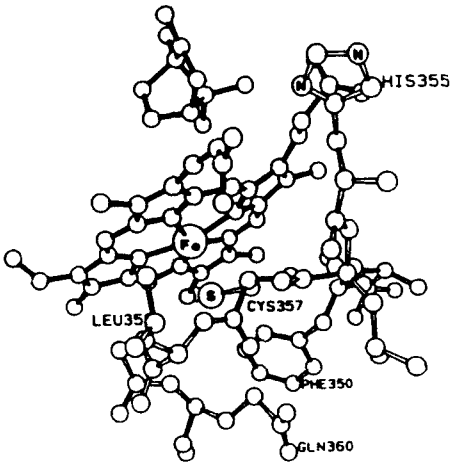


Figure 4: Cytochrome P-450 Active Site

The porphyrin molecule is a large, planar, aromatic, tetradentate, dianionic macrocycle which boasts four pyrrolic nitrogens directed inward on the molecule. These nitrogens possess the negative charge of the molecule and bind the positively charged metal ion within the center of the molecule. Iron, found in all cytochrome P-450 enzymes, is common in other heme enzymes, including hemoglobin and myoglobin. The periphery of the cytochrome P-450 porphyrin is asymmetric with

respect to functional groups, presumably in order to attach the metalloporphyrin to the protein chain in a particular orientation.

The Catalytic Cycle of Cytochrome P-450

As mentioned earlier, Cytochrome P-450 demonstrates an unusual ability to oxidize a variety of substrate molecules, including the extremely inert alkanes. The catalytic oxidation reaction of cytochrome P-450 follows the general form:

$$\text{Substrate} + 0_2 + 2e^-/2H^+ \text{ ---> } \text{Substrate(O)} + H_2O$$

This powerful oxidation reaction is thought to have the mechanism shown below in *Figure 5.* [12]

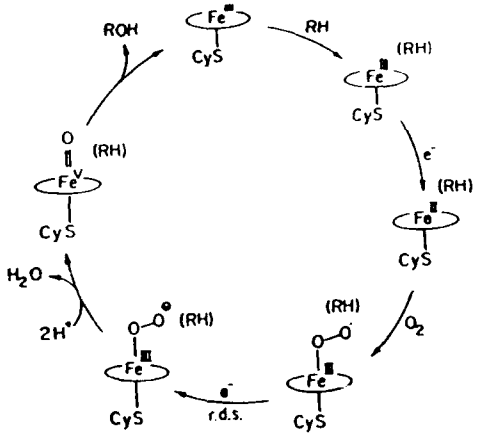


Figure 5: Catalytic Cycle of Cytochrome P-450

The porphyrin molecule of *Figure 4* has been represented by an ellipse for simplicity in *Figure 5.* The iron bound to the cysteine amino acid by the sulfur atom is still depicted. The protein (not pictured) binds the substrate in close proximity to the iron porphyrin in the low valent (+3 charge) resting state. This high spin ferric complex then gains an electron and is reduced to the (+2) state. Dioxygen is bound in an end-on fashion, and another electron is gained. This forms an iron (+3) peroxide complex. The intermediate molecules to this point in the catalytic cycle have been isolated and studied.[13] The intermediates after the rate determining step (r.d.s.) are too short-lived to be observed, and their identities are only conjecture. The next step adds two protons, cleaves the oxygen-oxygen bond heterolytically, and generates a molecule of water as a byproduct. With this step, the iron is oxidized to the unusual and highly reactive (+5) valent state with the monoxygen ligand bound to the iron. The exact structure of this high valent compound is still unknown, but it can be represented as either an oxoiron(V) species or an oxoiron(IV) porphyrin radical.[14] The substrate then accepts the oxygen atom from the iron and is ejected by the catalyst, which is once again in its resting (+3) low valent state, poised to oxidize more substrate molecules. The high valent oxoiron complex discussed above, which is responsible for the actual oxygen transfer to substrate, can also be prepared through a peroxide shunt reaction.[15] This shunt reaction uses an oxygen

rich donor molecule such as iodosobenzene or peroxides to form the oxygenated compex directly, bypassing the other steps in the catalytic cycle. It should be noted that the shunt reaction is carried out in the absence of molecular oxygen and reducing agent (source of electrons). This shunt reaction is utilized in several of the experiments contained within this study.

Model Studies

Model studies are often undertaken to study chemical systems that would otherwise be difficult to examine. Biological processes are prime examples of chemical systems that cannot be easily studied due to their delicacy and complexity. Therefore, if a model chemical system can be devised and substituted for the naturally occurring system in a representative manner, conclusions can be drawn regarding the natural system based on the chemistry of the model.

The modelling approach involves several premises.[16] First, the model must be representative of the native molecule in the particular property being examined. Second, the model must possess certain qualities that make it more convenient to study than the native molecule, such as stability in a laboratory setting. Preferably, the model compound would be inexpensive and easily obtained or synthesized.

In modelling enzymes, only the active sites must be represented. The effect of the protein holding the substrate molecule in position for reaction can be achieved by simply using a high concentration of the substrate. Simple probability replaces positional precision, and produces the same overall effect as the native system. This allows the protein parts of the enzyme to be left unmodelled.

A model study is only as good as the model used. The most difficult part of these studies is designing a model that is both representative of the native compound and easily studied in the laboratory. Some models are better than others and different models may provide different information about the same native compound.

In modelling the enzyme cytochrome P-450, there are several important points that must be considered. The uncommon thiolate ligand is not stable once removed from biological systems. Thus, the active site must be altered in some way to provide stability. Since the presumption is that the thiolate ligand is responsible for the powerful oxidative chemistry of cytochrome P-450, substituting for the sulfur atom would be pointless. The other alternative is to substitute for the iron atom. Rhodium was chosen to replace iron in the model studies described herein because it binds oxygen in the same end-on fashion as does iron. More importantly, rhodium forms more stable bonds with axial ligands than does iron, even coordinating with the sulfur atom.

The porphyrin ring of the native cytochrome P-450 is asymmetric, and thus difficult to synthesize. Assuming that the periphery of the porphyrin does not affect the oxidative chemistry of the molecule, an easily synthesized, symmetric porphyrin, tetraphenylporphyrin, was used in this model study.

In order to investigate the role of the thiolate bond in catalytic oxidations, the goal of this project, a series of rhodium porphyrins were synthesized. The only difference between member porphyrins of the series was the axial ligand attached to the rhodium metal. These ligands, which included thiolate, imidazole, acetate, and chloride, were chosen for their structural similarity to naturally occurring ligands. By comparing the results derived from the thiolate complex to results from the remaining members of the series, conclusions may be drawn as to the role of the thiolate ligand in the oxidative chemistry of cytochrome P-450. A diagram of the general model porphyrin,tetraphenylporphyrin, used in this study is shown below in *Figure 6.*

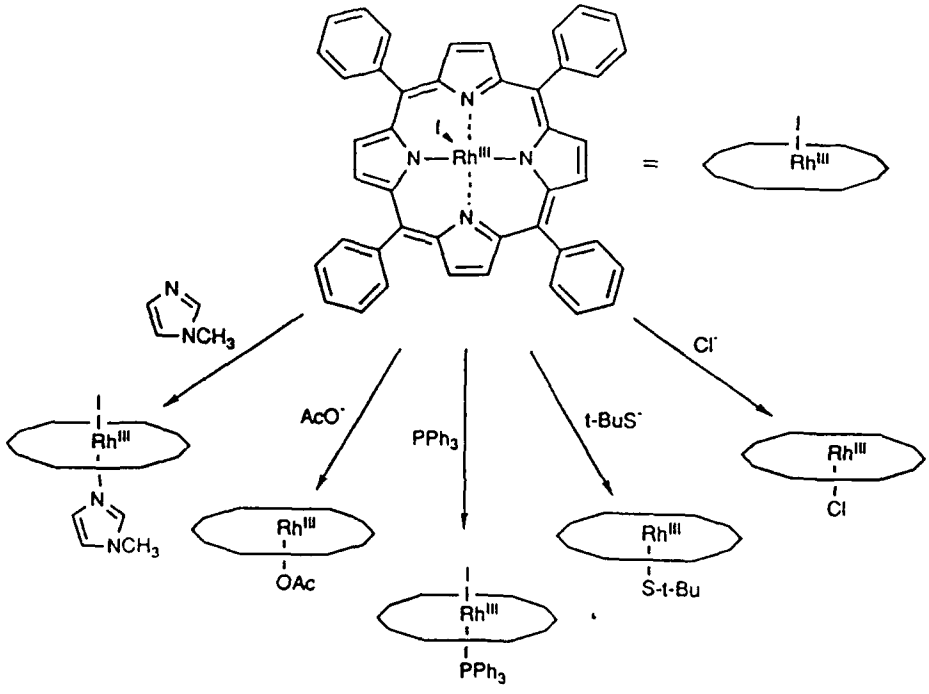


Figure 6: Synthetic Porphyrin and Ligands

Summary of Objectives

Cytochrome P-450 is currently being studied by many research groups because of its powerful oxidative chemistry and possible application to industrial and medical problems. This project has focused on the uncommon bond between iron and sulfur and determining the role this bond plays in the oxidation reaction as carried out by cytochrome P-450.

Statement of Approach

In approaching this study, there seemed to be three major possibilities for the role of the thiolate bond.[17] The first hypothesis held that the thiolate bond was in some way facilitating oxygen-oxygen bond cleavage (see step F->G in the catalytic cycle of *Figure 5*). The second stated that the thiolate bond might contribute to the stability of the high valent oxo porphyrin complex (see intermediate G in catalytic cycle) that actually effects the oxygen transfer between porphyrin and the substrate molecule. The final possibility was that the thiolate bond was moderating or directing the oxygen transfer to the substrate (see step G->A in catalytic cycle).

The experiments conducted in this study were designed to address the three possible roles mentioned above. Each experiment tested the validity of at least one of the hypotheses, and often more than one. There were four major experiments involved in this study. They included several catalytic reactions, high valent oxo complex formation and isolation, the kinetics of high valent oxo formation, and electrochemistry. Each experiment generated useful data in relation to the three possible roles of the thiolate ligand in the oxidative chemistry of cytochrome P-450.

Synthesis and Characterization

The series of rhodium porphyrins differing only in axial ligand were synthesized as per the experimental section (Appendix 1). The different porphyrins were characterized using ^{1}H NMR spectroscopy on a 300 MegaHertz GE instrument, and UV/Visible Electronic Spectroscopy on a Hewlett-Packard diode array spectrophotometer. The spectra characterizing the thiolate and the chloride analogues of the model compounds are given in *Figure* 7. The characteristic peaks of the ligands are shifted upfield in the ^{1}H NMR spectra due to the electron density that encircles the porphyrin ring. The electronic spectra show the characteristic rhodium porphyrin peaks, but shifted several wavenumbers depending on the ligand attached to the rhodium porphyrin.

Figure 7: Thiolate Complex NMR

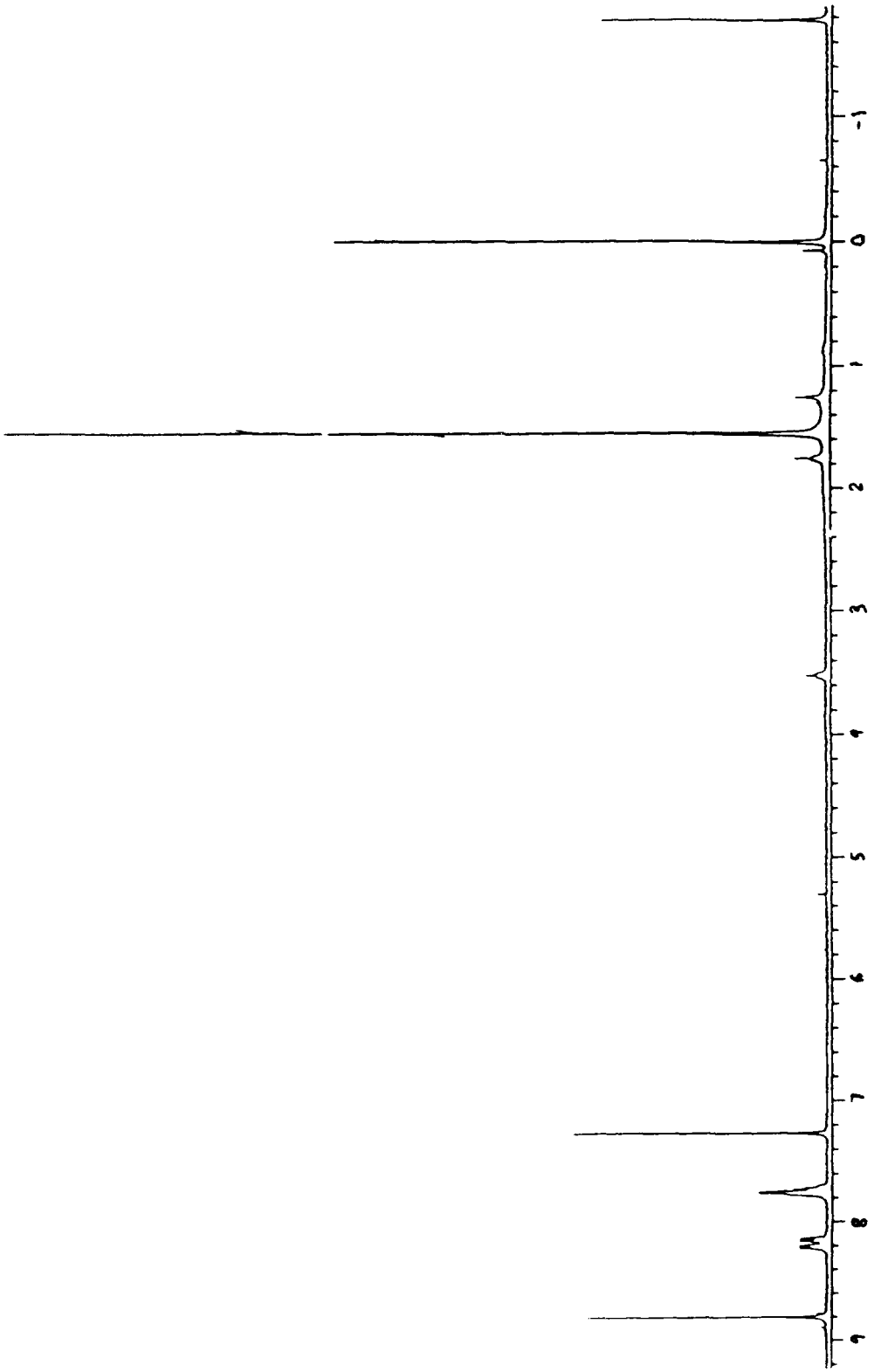


Figure 7: Thiolate Complex UV/Vis Spectrum

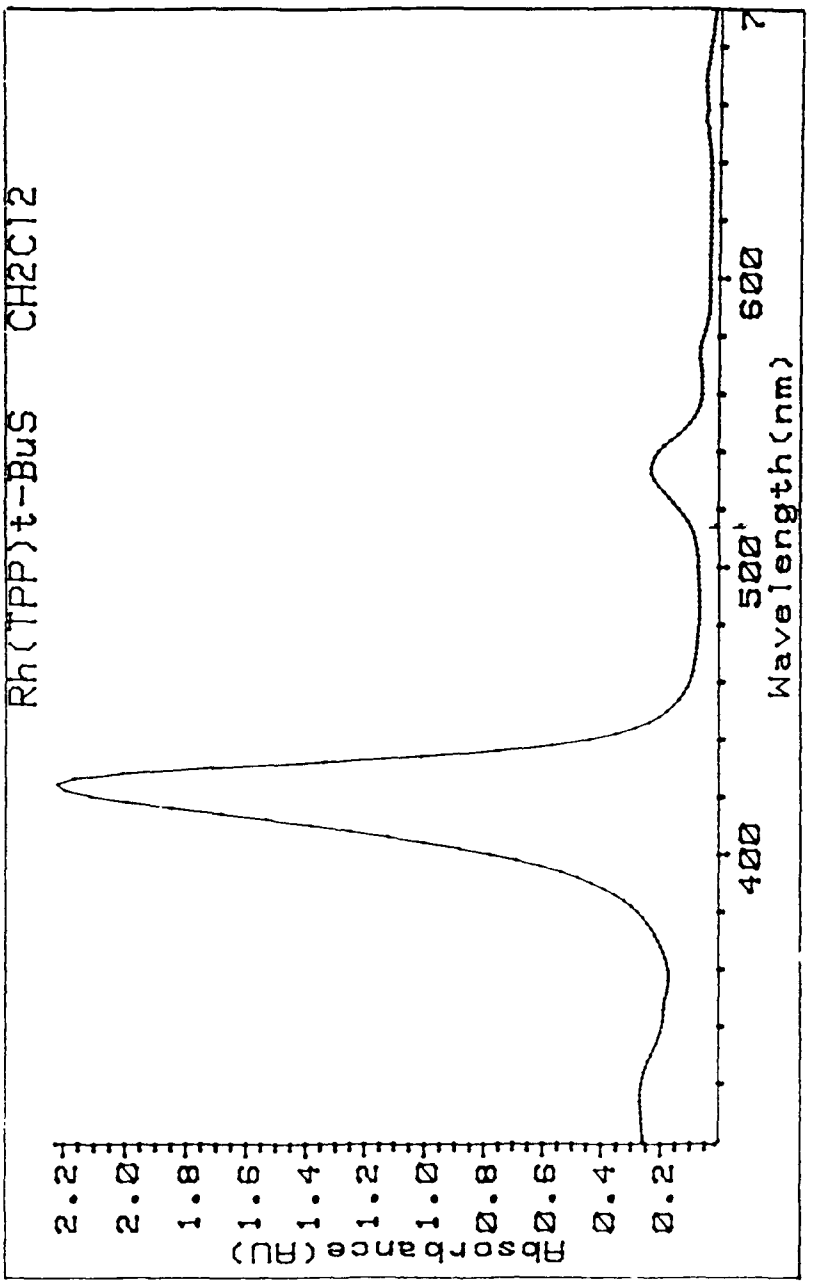


Figure 7: Chloride Complex NMR

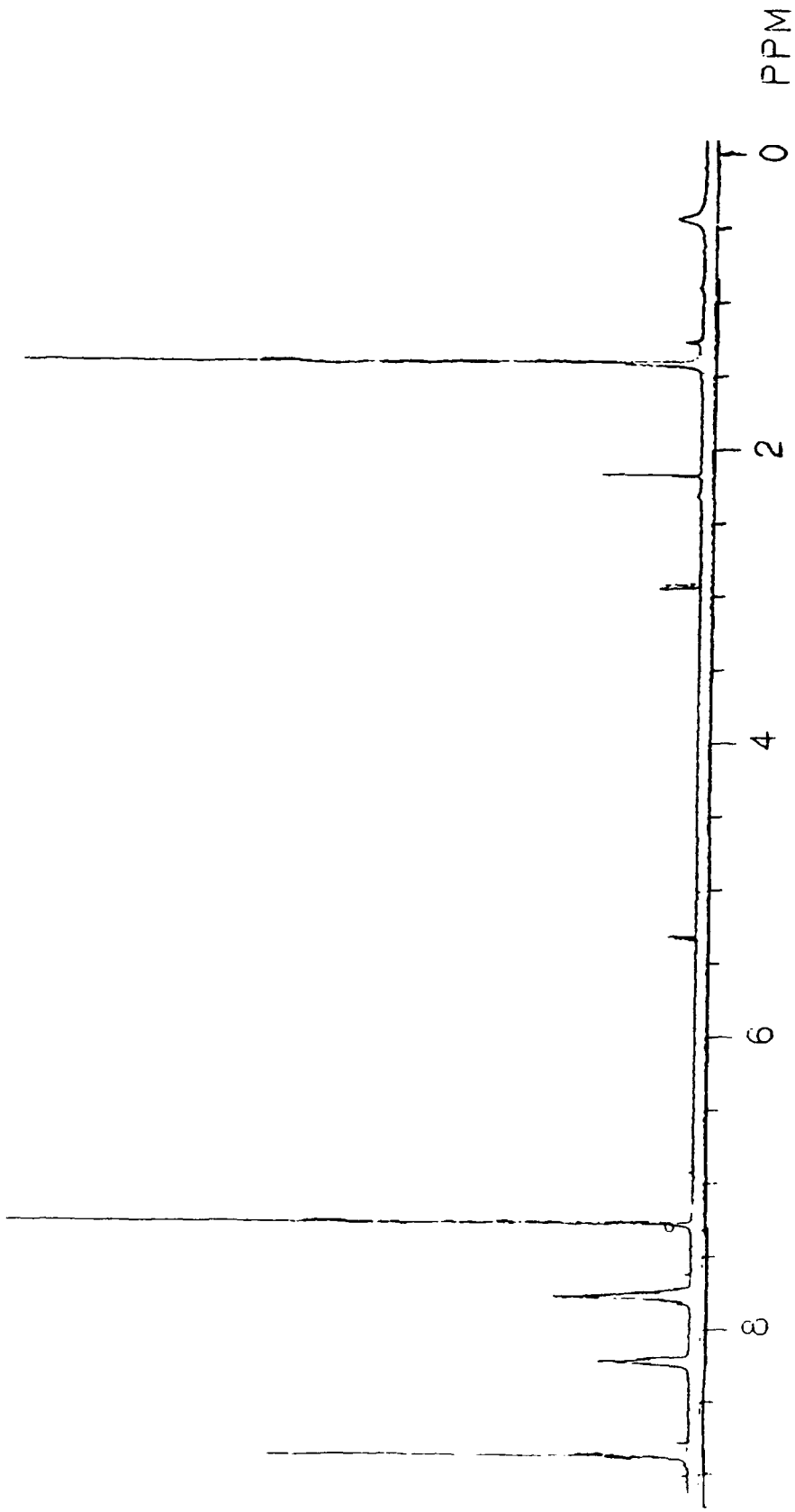
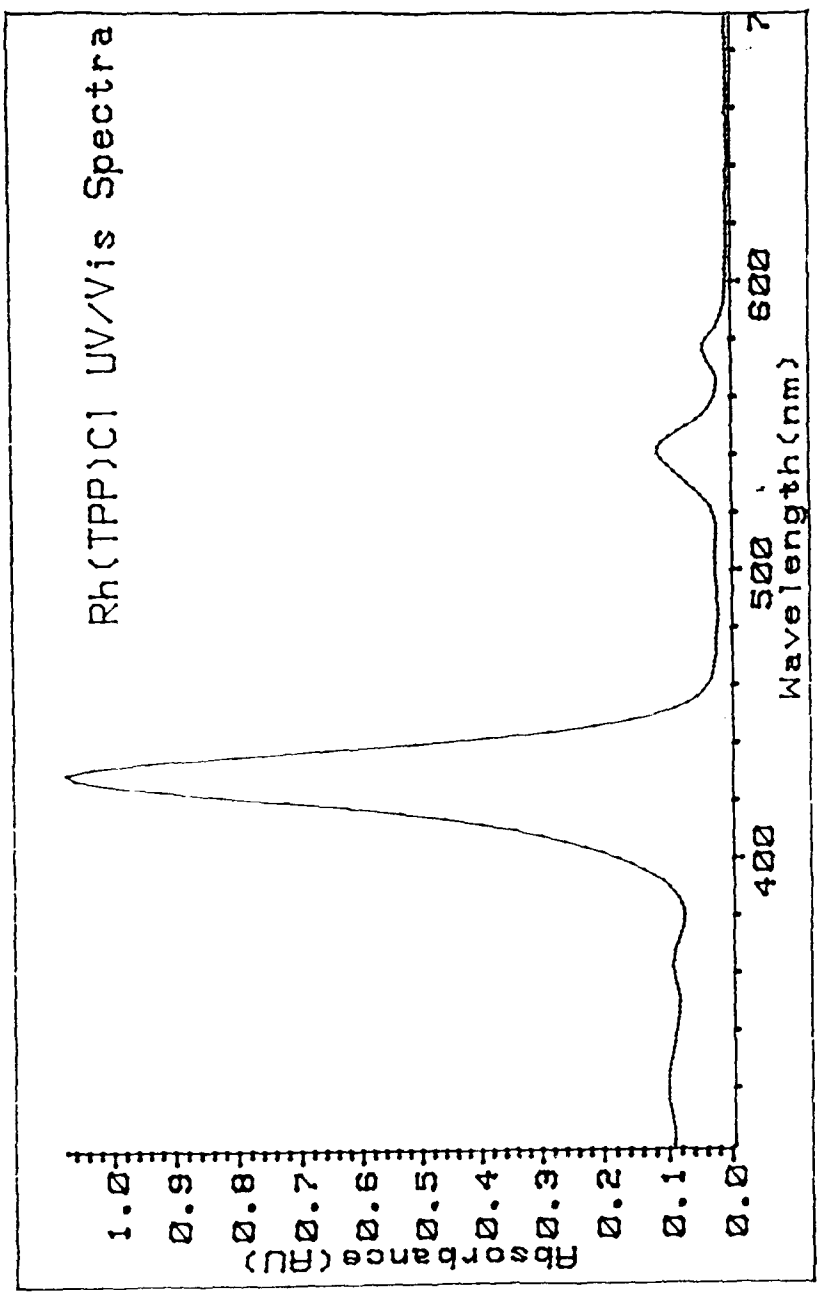
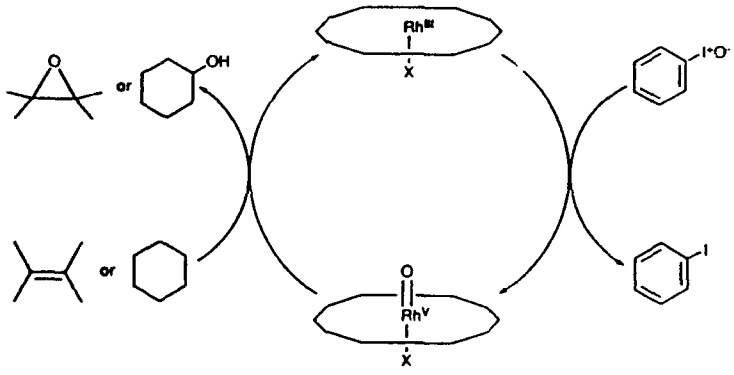


Figure 7: Chloride Complex UV/Vis Spectrum



Catalytic Reactions

Two major classes of oxidation reactions were examined using the model porphyrins as catalysts: epoxidation of alkenes and hydroxylation of alkanes. The specific reactions are shown in *Reaction Scheme 1* below.



Reaction Scheme 1: Catalytic Reactions

These reactions utilized the shunt reaction depicted in the catalytic cycle to produce the reactive high valent oxo complex. Iodosobenzene, an oxygen atom donor, transferred its oxygen to the porphyrin for this purpose.

The epoxidation reaction was carried out in dichloromethane using 2,3-dimethyl-2-butene as the substrate. The hydroxylation reaction was carried out in benzene using cyclohexane as the

substrate. The reactants and products were monitored through use of a gas chromatograph.

Mixtures of solvent, substrate, iodosobenzene, and an internal standard were prepared. In the absence of rhodium porphyrin, no reaction was observed. Upon addition of the porphyrin to heterogeneous solutions of iodosobenzene and 2,3-dimethyl-2-butene, the oxidation reaction occurred. At ten minute intervals, aliquots of reaction mixture were removed, centrifuged to remove unreacted oxidant, and examined by gas chromatography and UV/Vis spectroscopy. Steady growth in the amount of products was observed. The average yields, calculated by a ratio of product formed to oxidant used, were approximately 66% for the epoxidation and 3% for the hydroxylation. These values are acceptable for catalysis, although they seem to suggest that rhodium porphyrins are less efficient catalysts than similar iron porphyrins. The data obtained in this study as well as other studies substantiates this. (As a control, iron porphyrins with a chloride ligand attached were also run.)

Thus, the homologous series of rhodium porphyrins did in fact catalyze the epoxidation and hydroxylation reactions, demonstrating the ability to model the cytochrome P-450 shunt reaction. However, the data also demonstrated the fact that there was no dependence of oxidative power or efficiency on axial ligand identity; the thiolate-ligated porphyrin did not catalyze the reaction in higher yield or at a faster rate than the other

members of the series of porphyrins. This data, in relation to the three possible roles of the thiolate bond, suggests that the thiolate ligand does not facilitate or moderate oxygen atom transfer from the porphyrin to the substrate.

In addition, this experiment tested the stability of the oxo porphyrin complex as a function of axial ligand. Based on the UV/Vis spectra of the aliquots removed from the reaction mixture, the thiolate-ligated catalyst showed significant decomposition. Its spectrum decayed over one hour, whereas that of the other ligated porphyrins in the series showed no decay. The decomposition of the thiolate complex is shown in *Figure 8* below. The interval between spectra was ten minutes.

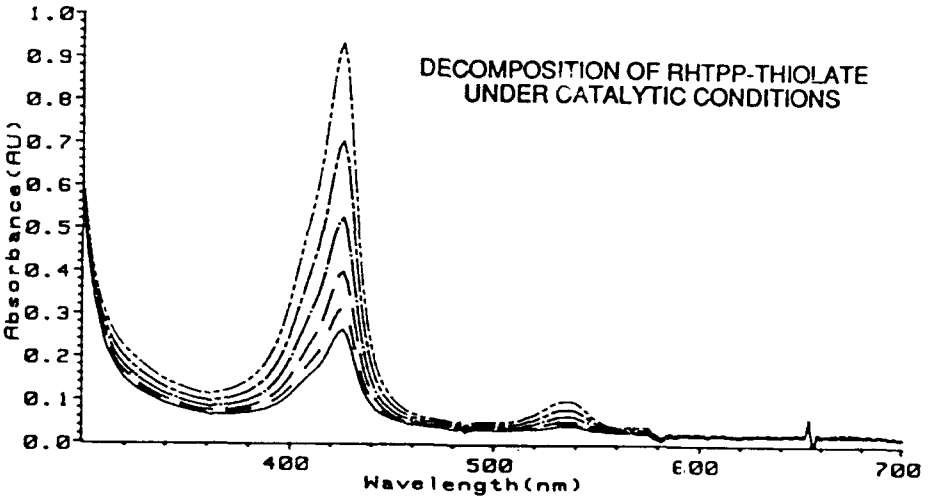


Figure 8: Decomposition of Thiolate Complex Over Time

This result is directly opposed to the second possible role of the thiolate ligand, namely that it stabilizes the high valent oxo complex. To the contrary, it seems the thiolate ligand destabilizes the high valent oxo complex.

Oxo Complex Formation and Isolation

The second major experiment of the study dealt with the formation and isolation of the high valent oxo porphyrin complex. This highly reactive intermediate has never been isolated, and its existence is still only hypothetical. The two structures that are acceptable with the current data are an oxo-iron porphyrin in the (+5) state and an oxo-iron porphyrin radical in the (+4) state.[18] Both are shown below in *Figure 9*.

Figure 9: Proposed Oxo Complex Structures

The shunt reaction of the catalytic cycle was again relied upon to promote the rhodium porphyrin to the high valent oxo complex (+5) state. In this experiment, *meta*-chloroperoxybenzoic acid (mCPBA) functioned as the oxygen atom donor. This oxidant is different from the iodosobenzene used previously in that it contains an O-O bond, which is broken as the reaction proceeds. Methanol was used as the solvent; the use of methylene chloride would have facilitated solubility of the porphyrins, but that solvent reacts with the high valent oxo complex.

The reaction was followed by UV/Vis spectroscopy, which indicated a shift in the characteristic peaks when the porphyrin changed oxidation states. An example of this shift is shown below in *Figure 10.*

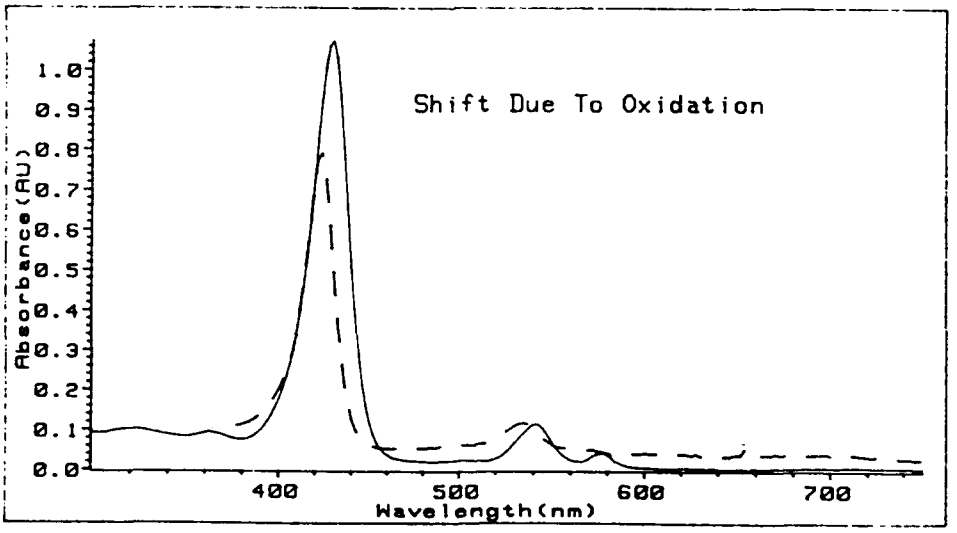


Figure 10: Spectral Shifts Upon Oxidation

The addition of exactly one equivalent of mCPBA oxidant (relative to amount of rhodium porphyrin) by syringe converted all the porphyrin to the high valent state. This result held true for all the porphyrins in the homologous series, although the characteristic shifts were of course different depending on ligand. This result again failed to distinguish the thiolate-ligated porphyrin as unique. It did indicate that the thiolate ligand is not involved in facilitating oxygen-oxygen bond cleavage, however, since the oxygen bond cleavage on the peracid (oxygen donor molecule) was not dependent on axial ligand identity.

Upon addition of a hundred-fold excess of 2,3-dimethyl-2-butene, there was no reaction noted as indicated by a reverse shift in the UV/Vis spectrum. This might be due to the methanol solvent, which apparently interferes with the reaction.

The thiolate-ligated porphyrin in the high valent oxo state was again found to be unstable over a period of 12 hours. It decomposed to yield the original rhodium (+3) porphyrin, whereas the porphyrins bearing the other ligands were stable in the high valent oxo state for greater than 48 hours. This again is in direct opposition to the second hypothesis, that the thiolate ligand stabilizes the oxo complex.

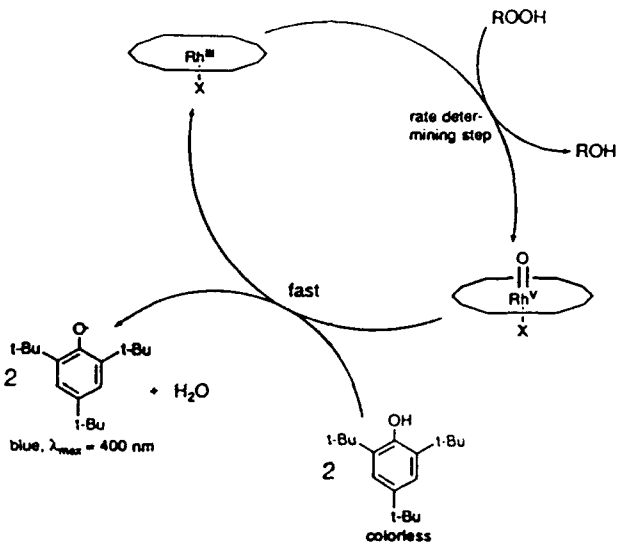
The isolation of the high valent oxo complexes was the other goal of this experiment. Once these complexes were formed (indicated by shift in visible spectrum), several attempts were

made to crystallize and isolate the highly reactive species. Standard recrystallization techniques, such as evaporation under air and removal of solvent by vacuum, were tried. Crystals were obtained in both cases; these were isolated, purified, and characterized. The identity of the crystals was determined by ^1H NMR to be the methoxy-ligated porphyrin in all cases. Somehow, the solvent molecules, present in great excess, had reacted with the complex and had replaced the ligand originally bound to the metal. Thus, the isolation of a high valent oxo porphyrin complex has still not been accomplished. The exact structure of this highly reactive molecule may never be known.

Kinetics of Oxo Formation

This experiment attempted to address the speed with which the high valent oxo porphyrin complex was formed and how the different porphyrins in the series varied with respect to this parameter. Kinetics experiments, those that deal with the speed of reactions, are often difficult to conduct. Special stopped-flow instruments for measuring very fast reactions are needed for some catalyst studies. Fortunately for this study, the rhodium porphyrin reactions were slow enough to follow by UV/Vis spectroscopy.

This study used an ingenious method for measuring the rate of high valent oxo rhodium porphyrin formation described by Traylor *et al.*[19] As the high valent oxo complex is formed through oxygen transfer from mCPBA, it immediately reacts with the substrate molecule, a substituted phenol. This substrate, which is originally colorless in solution, forms a blue colored product upon reaction with the rhodium oxo porphyrin complex. The rate determining step, or the slowest in the reaction process, is the cleavage of the oxygen-oxygen bond of the mCPBA and the transfer of oxygen to the porphyrin. The fast step, which is insignificant in the time realm of the rate determining step, is the oxygen transfer to the substrate. Thus, the production of the blue colored species is concurrent with cleavage of the oxygen-oxygen bond of mCPBA and formation of the high valent oxo complex. By configuring the computer controlled UV/Vis spectrometer to scan for the blue phenoxy radical product at regular intervals, the rate of reaction can be established. The reaction scheme of this experiment is shown below in *Reaction Scheme 2*.



Reaction Scheme 2: Kinetics of Oxo Formation

Mixtures of solvent, mCPBA, and tri-*tert*-butylphenol were prepared in a thermostatted cuvette. In the absence of porphyrin, no reaction ensued as indicated by the absence of blue color. Upon addition of the rhodium porphyrin, the reaction started. A typical plot of the production of blue tri-*tert*-butylphenoxy radical is shown below in *Figure 11* for the chloride analogue of the porphyrin series. The time interval between successive spectra is seven minutes.

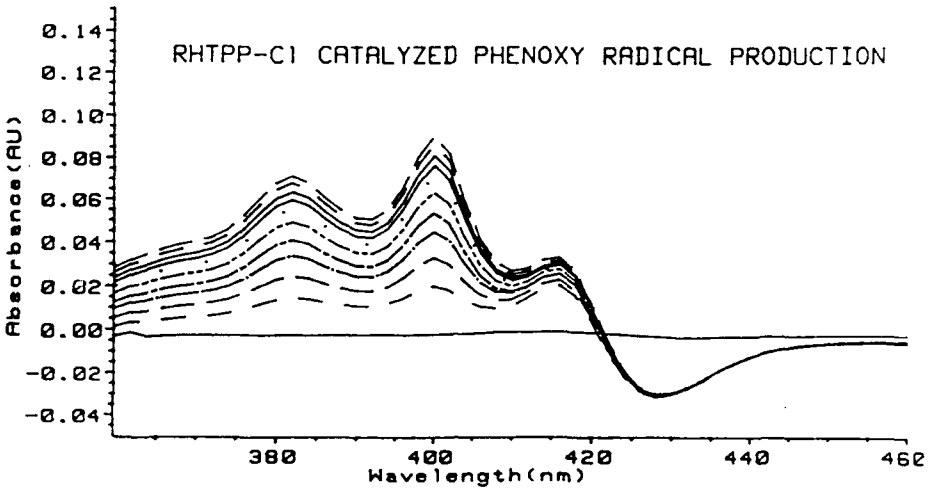


Figure 11: Production of Phenoxy Radical

The data obtained from the plots of absorbance at 400 nanometers with corresponding times can be used to calculate the rate constant for this reaction. By plotting the natural log of the absorbance at 400 nm at time ***t*** minus the absorbance at 400 nm at time infinity (when the reaction has proceeded to completion) versus the time ***t***, a straight line is obtained. The straight line indicates that the reaction is first order in terms of oxidant (mCPBA) and first order overall. The slope of that straight line, obtained by a least squares fit, represents the rate constant, ***k***, for the reaction. A typical plot is represented below in *Figure 12*. Notice the experimentally good linearity of the curve (correlation coefficient = .98).

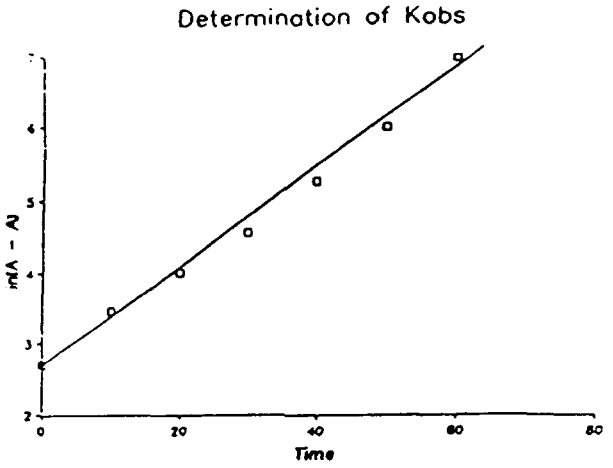


Figure 12: Determination of Rate Constant

The results of this experiment agree with the results from the catalytic reactions and the oxo formation experiments. The data showed no dependence of the reaction rate or rate constant on identity of axial ligand bound to the rhodium porphyrin. The thiolate-ligated porphyrin showed a similar growth curve and similar rate constant to the other porphyrins in the series. This relates to the original hypotheses in that the thiolate ligand does not seem to facilitate oxygen-oxygen bond cleavage. It does catalyze the reaction, but not differently than rhodium porphyrins coordinated by the other axial ligands in the series.

Electrochemistry

The final experiment in this study attempts to address the stability of the high valent oxo porphyrin complex. The other experiments have also provided data to this end, but this experiment was designed to show the stability (or instability) of the complex directly.[20]

The electrochemical technique known as cyclic voltammetry was used. Cyclic voltammetry uses an electrode placed in a solution containing the molecule of interest. The potential of the electrode is ramped such that it can either oxidize (take electrons away from) or reduce (add electrons to) a chemical species. The oxidation process associated with cyclic voltammetry (CV) is analogous to the addition of an oxygen atom to a molecule. By examining the reversibilty of an oxidation process (reduction), conclusions may be drawn as to the nature and behavior of the oxidized species.

The rhodium porphyrin to be examined is dissolved in methylene chloride, and the electrodes are placed in the solution. The electrode potential is scanned on the order of 100 millivolts per second. An oxidation occurs when there is a flow of current from the solution. An ammeter measures the current while a voltmeter monitors the electrode potential. The CV instrument then creates a plot of current versus voltage from the data

obtained. A dip in the plot indicates an oxidation has occurred. The direction of the potential is then reversed, and the oxidized product should be reduced (for a reversible process). A proper reversible process is one that demonstrates a reduction current flow at the same electrode potential as the dip of the oxidation process. An irreversible process is one that shows no corresponding current flow, indicating that some type of chemical reaction had occurred while the chemical species was in the oxidized state.

Cyclic voltammograms were obtained for the series of rhodium porphyrins. The CV's of the thiolate- and chloride-ligated rhodium porphyrins are shown below in *Figure 13*. The chloride ligated CV is representative of the other members of the rhodium porphyrin series. The results with regard to stability were consistent with the results obtained from the other experiments. The CV of the thiolate-ligated rhodium porphyrin demonstrated a distinct irreversible reduction process, whereas the other members of the series showed reversible processes. The thiolate ligand destabilizes the high valent porphyrin complex, again in contradiction to the second possible role that the thiolate ligand might have played in the catalytic cycle of tochrome P-450.

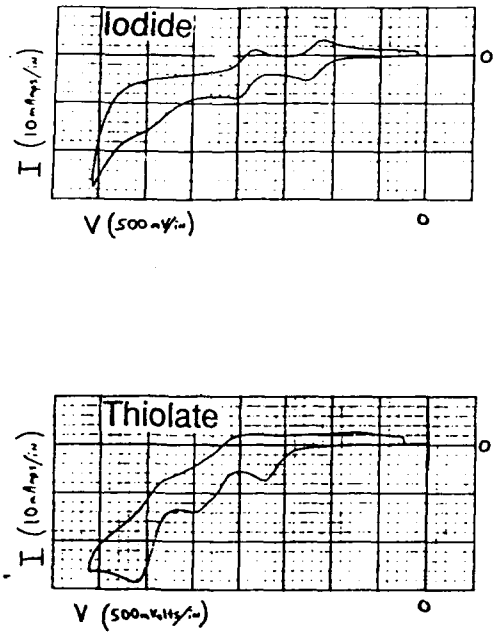


Figure 13: Cyclic Voltammograms

Conclusions

This study has addressed the role of the unique thiolate ligand in the unusual oxidative chemistry associated with the enzyme cytochrome P-450. Due to the inherent instability of the iron sulfur bond, a rhodium tetraphenyl porphyrin was chosen to model the iron protoporphyrin IX found in the native enzyme. The rhodium porphyrin performed as expected by forming an air stable bond with a sulfur ligand. The synthesis of a metalloporphyrin thiolate complex is significant; very few such complexes are reported in the literature. The model was representative of the native enzyme in that it did in fact catalyze oxidative reactions

similar to those of the enzyme. The rhodium model porphyrin was significantly less efficient a catalyst, though.

The possible roles of the thiolate ligand that were to be examined in this study included the possibility of facilitating oxygen-oxygen bond cleavage, stabilizing the high valent oxo porphyrin complex, and moderating the oxygen transfer from porphyrin to substrate molecule. Interestingly, there was no evidence gathered that supported any of these possible roles. In addition, there was overwhelming evidence that actually contradicted the second of the three possible roles, namely that the thiolate ligand stabilized the high valent oxo porphyrin complex. Three of the four experiments conducted illustrated the destabilizing effect that the thiolate ligand had on high valent porphyrin stability.

These conclusions are based on the assumption that the rhodium porphyrin complexes studied here are representative of the cytochrome -450 active site. The fact that these rhodium porphyrins will catalyze "P-450 like" shunt reactions suggests that our conclusions are valid.

However, one of our conclusions has been called into question by a recent report in the literature. Higuchi et al. synthesized an excellent model for the cytochrome P-450 enzymes that includes a thiolate ligand to an iron atom.[21] They overcame the instability of the iron-sulfur bond by attaching the other end of the thiolate

ligand to the periphery of the porphyrin ring structure. See *Figure 14* for this molecule.

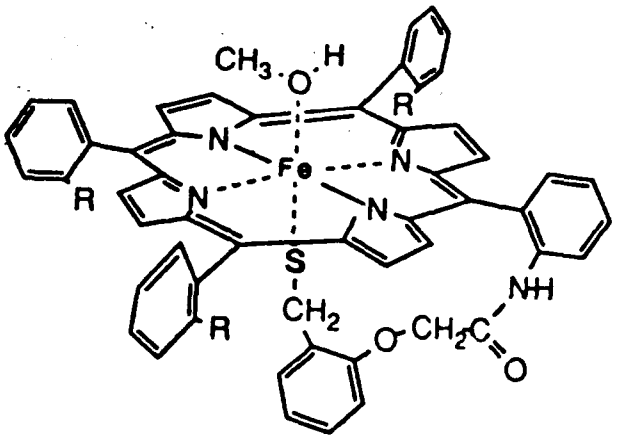


Figure 14: Higuchi Model Complex

Their study achieved data that was contradictory to our conclusion regarding the effect of the thiolate ligand on oxygen-oxygen bond cleavage. They achieved a 250 fold increase in the rate of oxygen-oxygen bond cleavage when catalyzed by chloride- versus thiolate-ligated iron porphyrins. As was stated in the description of model studies above, some models are better than others and different models may provide different information. This is a good example of these points.

Thus, although we now have some clues, the question still remains. What advantage does the thiolate ligand lend to the cytochrome P-450 enzymes? This model study has attempted to address these concerns, but has succeeded in addressing only one

- and in a negative manner. Our results suggest it is not stabilization of the high valent oxo intermediate that gives cytochrome P-450 its chemistry. Recent results from Higuchi suggest it is to facilitate oxygen-oxygen bond cleavage. These conclusions must be taken into account when attempts are made to find an industrially applicable catalyst which will mimic the properties of cytochrome P-450.

List of Figures

1 Sythesis of Nylon 6
2 Biosynthesis of Sex Hormones 8
3 Enzyme Structure 12
4 Cytochrome P-450 Active Site 13
5 Catalytic Cycle of Cytochrome P-450 14
6 Synthetic Porphyrin and Ligands 19
7 Spectra 22
8 Decomposition of Thiolate Complex Over Time 28
9 Proposed Oxo Complex Structures 29
10 Spectral Shifts Upon Oxidation 30
11 Production of Phenoxy Radical 35
12 Determination of Rate Constant 36
13 Cyclic Voltammograms 39
14 Higuchi Model Compound 42

Reaction Scheme 1 26
Reaction Scheme 2 34

References

1 Solomons, T.W. *Organic Chemistry, 4th Ed.*; Wiley, Toronto, 1988; Chap. 3.

2 Williamson, *Macroscale and Microscale Organic Experiments*; Heath, New York, 1989; Chap. 5, p.71.

3 Stryer, L. *Biochemistry*, 2nd Ed.; Freeman, New York, 1975; Chap. 20, p.476.

4 Bender et al. *Bio-organic Chemistry of Enzymatic Catalysis;* Wiley, Toronto, 1984; p.3.

5 Stryer, L. Chap. 4, p.65.

6 Stryer, L. Chap. 18, p.433.

7 Ortiz de Montellano, P.R. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry;* Ortiz de Montellano, P.R., Ed.; Plenum Press, New York, 1986; p. vii.

8 *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; p.vii.

9 Poulos, T.L. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P.R., Ed.; Plenum Press, New York, 1986; p.516.

10 Groves et al. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry;* p.6.

11 Sligar et al. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry;* p. 461.

12 Groves et al. p.2.

13 Groves et al. p.3.

14 Groves et al. p.3.

15 Groves et al. p.2.

16 Shriver. *Inorganic Chemistry* ; Freeman, New York, 1990; Chap. 19, p.612.

17 Valentine, J; American Chemical Society Meeting, Los Angeles, Sept. 25-30, 1988.

18 Groves et al. p.3.

19 Traylor et al. *J. Am. Chem. Soc.* **1984**, *106*, 755-764.

20 Kadish et al. *Inorg. Chem.* **1985**, *24*, 4515-4520.

21 Higuchi et al. *J. Am. Chem. Soc.* **1990**, *112*, 7051-7053.

22 Adler et al. *J. Org. Chem.* **1967**, *32*, 476.

Appendix 1: Experimental Section

Instrumentation

A GE QE-300 NMR instrument was used to characterize all species synthesized. A Hewlett-Packard diode array UV/Vis instrument was used to monitor formation of oxo complexes and the kinetics of those formations. A Bioanalytical Systems wave generator and electrode were used in the cyclic voltammetry studies.

Materials

Reagent grade solvents were used in all synthetic and analytical steps, and were purified as described in the procedures below. mCPBA was recrystallized from ether/petroleum ether. Tri-*tert*-butylphenol was recrystallized from ethanol. Deuterated solvents were obtained from Aldrich. All other organic reagents were purchased at the highest level of purity available.

Synthesis

Tetraphenylporphyrin H_2(TPP)

Tetraphenylporphyrin was prepared using the method described by Adler et al.[22] Pyrrole (18.6 mL, 0.27 mol), which had been passed through neutral alumina, and reagent grade benzaldehyde (26.6 mL, 0.27 mol) were added to one liter of freshly distilled, refluxing propionic acid. The solution was refluxed for one half hour, during which time it gradually darkened to a deep purple color. The solution was cooled to room temperature, filtered, and washed consecutively with cold methanol and hot water. Purple crystals were obtained after drying under vacuum.
Yield 6.5 g (16%).

The crude porphyrin is often contaminated with chlorin impurities. This incomplete oxidation is noticed upon examination of the ^{1}H NMR spectrum, which does not show the typical porphyrin signals. When this was the case, a 10 mL of a 10% solution of DDQ in methylene chloride was added, and stirred at reflux for 30 minutes.

The tetraphenylporphyrin was purified via flash chromatography on silica gel. The eluent used was an increasing gradient of petroleum ether ----> methylene chloride.

UV/Vis (CH_2Cl_2): λ_{max} 418, 514, 548, 588 nm
^1H NMR ($CDCl_3$): δ 8.84 (s,8H), 8.23 (d,4H), 8.20(d,4H), 7.77 (m,12H)

Rhodium tetraphenylporphyrin iodide, Rh(TPP)I

Unmetallated tetraphenylporphyrin (1.00 g, 2.00 mmol) was dissolved in 110 mL methylene chloride that had been passed through neutral alumina. To this solution was added finely ground anhydrous sodium acetate (2.5 g, 0.03 mol). Next, rhodium(I) dicarbonyl chloride was added, at which time the solution showed a visible change in color. The reaction, followed by UV/Vis spectroscopy, was completed after 15 minutes. The excess salts were removed by washing three times with 150 mL aliquots of water. The solution was then dried over anhydrous sodium sulfate, filtered, and the solvent removed on a rotovap. This crude metallated tetraphenylporphyrin, known as a 'sitting atop complex', was then dissolved in benzene and treated with a solution of iodine crystals (0.4 g, 3.2 mmol) dissolved in 50 mL benzene. (Note: using too much iodine (1.0 g) oxidized the 'sitting atop complex' so much that it was not usable). The oxidation with

iodine was also followed by UV/Vis spectroscopy, and after five minutes, the spectrum changed as below to indicate the formation of the metallated rhodium tetraphenylporphyrin iodide molecule.

The rhodium tetraphenylporphyrin iodide was isolated and purified using a flash chromatography technique. The Rh(TPP)I was first adsorbed onto 50 mL silica gel and dried under high vacuum. The adsorbed silica gel was slurried with petroleum ether and placed on top of a silica gel flash chromatography column (approximately 80 mL or four inches in 1.5 inch diameter column). The desired compound was eluted and collected as a deep red band using a solvent gradient of petroleum ether----> methylene chloride.

UV/Vis (CH_2Cl_2): sitting atop complex, λ_{max} 372, 418, 458 nm
Rh(TPP)I, λ_{max} 412, 458 nm
^1H NMR ($CDCl_3$): δ 8.87 (s,8H), 8.21(d,4H), 8.13(d,4H), 7.75(m,12H)

Rhodium tetraphenylporphyrin chloride, Rh(TPP)Cl

In chloroform, Rh(TPP)I (300 mg, 0.4 mmol) was stirred at reflux with trimethylbenzylammonium chloride (700 mg, 1.0 mmol) for 15 minutes. The solution was then cooled to room temperature and washed three times with 100 mL aliquots of water to remove any excess salts. After drying the solution over anhydrous sodium sulfate and filtering, the chloride analogue was

adsorbed onto 50 mL of flash grade silica gel, dried under high vacuum, and loaded on a flash chromatography column. The orange-red band was collected using a solvent gradient of petroleum ether ----> methylene chloride. Pure Rh(TPP)Cl was recrystallized by layering hexanes onto a benzene solution of the porphyrin.

UV/Vis (CH_2Cl_2): λ_{max} 412, 426 nm
^1H NMR ($CDCl_3$): δ 8.82(s,8H), 8.24(d,4H), 8.14(d,4H), 7.73(m,12H)

Rhodium tetraphenylporphyrin t-butylthiolate, Rh(TPP)-S-t-Bu

(Note: to avoid the unpleasant and alarming odor of the thiol compound, the reaction flask was attached to a bubbler system containing a 10% NaOH solution.)

The t-butylthiolate analogue of Rh(TPP) was prepared in an oxygen free atmosphere. In a 100 mL flask fitted with a nitrogen inlet tube, KH (30 mg, 0.75 mmol) was added to 25 mL of anhydrous THF. t-Butylthiol (0.25 mL, 0.5 mmol) was then added to the solution, which bubbled with the evolution of hydrogen gas. After the deprotonation reaction was complete, rhodium tetraphenylporphyrin iodide (20 mg, 0.025 mmol) was added, and the solution stirred at reflux for 30 minutes. The solution was

then cooled to room temperature, and 10 mL of water were added to dissolve any inorganic salts. Rh(TPP)-t-butylthiolate appears as a red-pink precipitate on the addition of the water to the solution. The THF is then removed on the rotovap, the precipitate collected over a Buchner funnel and washed thoroughly with water. After drying the t-butylthiolate complex under high vacuum, the crystals were dissolved in benzene and recrystallized by vapor diffusion of hexanes into the benzene solution.

UV/Vis (CH_2Cl_2): λ_{max} 412, 526 nm
^1H NMR ($CDCl_3$): δ 8.79(s,8H), 8.20(d,4H), 8.15(d,4H), 7.76(m,12H) -1.78(s,9H)

Rhodium tetraphenylporphyrin acetate, Rh(TPP)OAc

In a 100 mL flask fitted with a nitrogen inlet tube, KH (30 mg, 0.75 mmol) was added to 25 mL of anhydrous THF. Acetic acid (0.25 mL, 4.38 mmol) was then added to the solution, which bubbled with the evolution of hydrogen gas. After the deprotonation reaction was complete, rhodium tetraphenyl-porphyrin iodide (20 mg, 0.025 mmol) was added, and the solution stirred at reflux for 30 minutes. The solution was then cooled to room temperature, and 10 mL of water were added to dissolve any inorganic salts. Upon addition of the water, a red precipitate

appears. The THF is removed on the rotovap and the precipitate is collected and washed generously with water. The dried crystals are dissolved in benzene and recrystallized by vapor diffusion of hexanes into the benzene solution.

UV/Vis (CH_2Cl_2): λ_{max} 414, 526 nm
^1H NMR ($CDCl_3$): δ 8.83(s,8H), 8.22(d,4H), 8.17(d,4H), 7.75(m,12H), 0.08(s,3H)

Rhodium tetraphenylporphyrin iodide 1-methylimidazole, Rh(TPP)I(1MeIm)

To a solution of Rh(TPP)I (100 mg, 0.13 mmol) dissolved in 50 mL methylene chloride was added 1-methylimidazole (0.25 mL, 3.2 mmol). The solution changed from a deep purple color to a medium red color within three minutes. The solution was washed free of excess 1-methylimidazole with 10 mL water, and dried over anhydrous sodium sulfate. The solvent was removed on the rotovap, and the resulting crystals dried under high vacuum.

UV/Vis (CH_2Cl_2): λ_{max} 364, 430, 542, 576 nm
^1H NMR ($CDCl_3$): δ 8.83(s,8H), 8.21(d,4H), 8.14(d,4H), 7.72(m,12H), 4.61(s,1H), 2.10(s,3H), 1.04(s,1H), 0.62(s,1H)

Rhodium tetraphenylporphyrin iodide triphenylphosphine, $Rh(TPP)I(PPh_3)$

To a solution of Rh(TPP)I (100 mg, 0.13 mmol) dissolved in 50 mL methylene chloride was added triphenylphosphine (100 mg, 0.38 mmol). The deep purple solution became less intense on the addition. The solution was then adsorbed onto silica gel and loaded onto a flash chromatography column in order to remove any excess triphenylphosphine. The deep red band was collected by a solvent gradient of methylene chloride ----> 4% diethyl ether/methylene chloride. The solvent was removed by rotovap and the resulting crystals dried under high vacuum.

UV/Vis (CH_2Cl_2): λ_{max} 378, 450, 558, 596 nm
^1H NMR ($CDCl_3$): δ 8.70(s,8H), 8.07(d,4H), 7.84(d,4H), 7.70(m,12H), 6.96(t,3H), 6.55(t,6H), 3.92(t,6H)

Experimental Procedures

Catalytic Reactions

In 4 mL of methylene chloride, the following reagents were placed: 200 mg iodosobenzene, 400 µL 2,3-dimethyl-2-butene (or cyclohexane), and 50 µL octane, the internal standard. To this stirring mixture, 1.0 mg rhodium porphyrin was added. Gas chromatography of aliquots taken every 15 minutes were obtained

to monitor production of epoxide (or alcohol) and loss of oxidant. Aliquots were first centrifuged to remove any excess oxidant present.

Oxo Formation and Isolation

Exactly 5 mg rhodium porphyrin were dissolved in 300 mL of degassed methanol. $3x10^{-3}$ M solutions of mCPBA in methanol were prepared and titrated against the porphyrin solutions. The formation of the high valent oxo complex was followed by shifts in the UV/Vis spectra. Attempts to isolate these new complexes were made, including vacuum and air evaporations of the solvent.

Kinetics of Oxo Formation

Solutions .11 M in tri-*tert*-butylphenol and $1.1x10^{-6}$ M in rhodium porphyrin were prepared in degassed methanol. To keep the volume and temperature constant, the reactions were carried out in capped cuvettes and in an isothermal cuvette holder. The UV/Vis instrument was configured to obtain spectra every minute. A solution $1.0x10^{-3}$ M in mCPBA was prepared in degassed methanol and added to the other reactants in the cuvette. Solutions of $1.0x10^{-3}$ M and $1.0x10^{-1}$ M t-butyl-hydroperoxide were also tried as oxidants.

Electrochemistry

Cyclic voltammetry experiments were conducted in 10 mL CV flasks with reference, working, and auxiliary electrodes. 4 mg of rhodium porphyrin were dissolved in 7 mL of a .2 M solution of the electrolyte tetrabutylammonium tetrafluoroborate. The voltage was applied by scanning through one volt at a rate of 100 mVolts/second.